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β-cell specific T-lymphocyte response has a distinct inflammatory phenotype in children with Type 1 diabetes compared with adults

Short title: β-cell specific CD4 responses in children and adults

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What's new?

- Type 1 diabetes development in children appears more rapid and severe compared with that in adults. This paper shows that immune responses against β cells are more common and target more autoantigens in children compared with adults. In addition, the immune response in children is particularly focused on proinsulin and insulin as the main drivers of the autoimmune response.
- The findings of this study suggest age-related immunological heterogeneity in Type 1 diabetes. This may be important in developing age-appropriate immune-intervention strategies.

Abstract

Aim To examine the hypothesis that the quality, magnitude and breadth of helper T-lymphocyte responses to β cells differ in Type 1 diabetes according to diagnosis in childhood or adulthood.

Methods We studied helper T-lymphocyte reactivity against β-cell autoantigens by measuring production of the pro-inflammatory cytokine interferon-γ and the anti-inflammatory cytokine interleukin-10, using enzyme-linked immunospot assays in 61 people with Type 1 diabetes

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(within 3 months of diagnosis, positive for HLA *DRB1*0301* and/or **0401*), of whom 33 were children/adolescents, and 91 unaffected siblings.

Results Interferon-γ responses were significantly more frequent in children with Type 1 diabetes compared with adults (85 vs 61%; P=0.04). Insulin and proinsulin peptides were preferentially targeted in children (P= 0.0001 and P=0.04, respectively) and the breadth of the interferon-γ response was also greater, with 70% of children having an interferon-γ response to three or more peptides compared with 14% of adults (P<0.0001). Islet β-cell antigen-specific interleukin-10 responses were similar in children and adults in terms of frequency, breadth and magnitude, with the exception of responses to glutamic acid decarboxylase 65, which were significantly less frequent in adults.

Conclusions At diagnosis of Type 1 diabetes, pro-inflammatory autoreactivity is significantly more prevalent, focuses on a wider range of targets, and is more focused on insulin/proinsulin in children than adults. We interpret this as indicating a more aggressive immunological response in the younger age group that is especially characterized by loss of tolerance to proinsulin. These findings highlight the existence of age-related heterogeneity in Type 1 diabetes pathogenesis that could have relevance to the development of immune-based therapies.

Introduction

The incidence of Type 1 diabetes has increased worldwide during the last decade, especially in children [1] who typically develop disease with more severe and rapid onset of symptoms than adults [2]. This clinical observation might have several different explanations or be attributable to a combination of effects. The possibility that it reflects a different disease tempo in children compared with adults, resulting, in turn, from differences in the autoimmune response, is an attractive and important notion, as it would potentially influence

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strategies for the deployment of immunological interventions. For example, children might be treated at an earlier stage in the disease process, more aggressively or with a different set of therapeutic agents if it transpired that their autoimmune response has a different quality or magnitude compared with adults.

Comparative studies on β-cell-specific autoimmunity around the time of diagnosis in adults and children are scant, but studies conducted in the setting of childhood-onset Type 1 diabetes indicate that there may be within-disease, age-related effects on some aspects of autoimmunity, notably autoantibodies. For example, the appearance of autoantibodies within the first 2 years of life is usually accompanied by the development of multiple specificities [3] and rapid progression to Type 1 diabetes; in contrast, children who develop autoantibodies later have a slower progression to multiple autoantibodies and disease [4]. The relationship of these age-related differences in autoantibodies to the tempo of Type 1 diabetes development, however, is difficult to gauge because antibodies are not considered to be directly responsible for β-cell damage. Rather, it is generally proposed that CD4 and CD8 T-lymphocytes act in concert to destroy β cells, through a combination of inflammatory mediators and direct cytotoxicity, with β-cell-specific CD4 T-lymphocytes (also known as helper T-lymphocytes) as the main orchestrators of the process [5,6]. Thus, the current lack of comparative data on autoreactive helper T-lymphocytes in children and adults developing Type 1 diabetes represents a significant knowledge gap that potentially affects the translation of new intervention strategies into paediatric clinics.

We have previously shown the existence of disease endotypes in Type 1 diabetes [7] based on heterogeneity in both the adaptive immune response and islet pathology; and have now sought to extend these findings by examining the frequency, magnitude, breadth or quality of the β-cell-specific helper T-lymphocyte responses that prevail at diagnosis of Type 1 diabetes in children and adults.

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Materials and methods

Subjects and autoantibodies

Between 2009 and 2012 fresh heparinized blood was obtained from 61 people with newly diagnosed Type 1 diabetes [duration ≤ 12 weeks; 33 children/adolescents (defined as aged ≤16 years); 28 adults] and from 91 of their autoantibody-negative siblings without Type 1 diabetes (48 children; 43 adults; Table 1). Children and adults with Type 1 diabetes did not differ significantly with regard to disease duration, gender distribution, frequency of HLA genes, HbA_{1c} level or autoantibody prevalence (Table 1). The studies were approved by the National Research Ethics Service and informed consent was obtained from all participants/parents/guardians. Participants were enrolled if they possessed one or both of *HLA-DRB1*0301* and *HLA-DRB1*0401* (Table 1). Autoantibodies to glutamic acid decarboxylase 65 (GAD65), intracytoplasmic (606–979) islet antigen 2 (IA-2) and zinc transporter 8 (ZnT8) were measured by radioimmunoassay [8,9]. Insulin autoantibodies were not tested. The present work is an extension to our previous study showing the existence of disease endotypes in Type 1 diabetes [7].

Measuring β-cell-specific cytokine secreting $CD4^+$ T cells

Peptides representing naturally processed and presented IA-2, proinsulin and GAD65 epitopes, and overlapping regions of the insulin B and A chain, were used as stimuli at a concentration of 10 μg/ml to stimulate 2×10^6 cells [7]. Pediacel penta-vaccine (Sanofi Pasteur Ltd, Maidenhead, UK) was used at 1 μl/ml to examine anamnestic responses induced by vaccination or infection. Interferon (IFN)-γ and interleukin (IL)-10 production by $CD4^+$ T cells was detected by enzyme-linked immunospot assay, performed as described in the TrialNet T cell Validation blinded workshop, in triplicate for each peptide, and data were

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expressed as stimulation index (SI) values; SI values ≥3 were taken to indicate a positive response [7].

The interassay coefficient of variation was evaluated by repeated measurement of spot numbers to recall antigens using the same donor over a 12-month period. The coefficients of variation for the spot number for both the IFN-γ and IL-10 assays were 12.3 and 10.7%, respectively.

Statistical analysis

Positive responses were compared using Fisher's exact test. T-cell response data were aggregated for an autoantigen (proinsulin, insulin, GAD65, IA-2) and if any of the derivative peptides elicited a response, this autoantigen was considered positive [7].

Results

Interferon-γ responses in children and adults with newly diagnosed Type 1 diabetes

A higher frequency of children with Type 1 diabetes (28/33; 85%) showed an IFN-γ response to one or more of the islet-autoantigen peptides compared with adults [17/28, 61%; P=0.04, (Fig. 1a)]. Two findings suggest that this difference did not simply reflect a difference in age. First, we found that children who were autoantibody-negative siblings without Type 1 diabetes had significantly lower IFN-γ responses (12/48, 25%) compared with children (P=0.0001) and adults (P=0.003) with Type 1 diabetes; autoantibody-negative adult siblings also had significantly lower IFN-γ responses (13/43, 30%) compared with adults (P=0.015) and children (P<0.0001) with Type 1 diabetes. Second, the prevalence of anamnestic IFN-γ responses to pentavalent vaccine was similar in children and adults (96 and 94%, respectively; Table 1). There were no gender biases in the detected responses.

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The specificity of IFN-γ response also differed between children and adults, notably to insulin and proinsulin peptides. Responses to insulin A1-21 (33% in children vs 7% in adults; *P*=0.025), insulin B1-20 (42 vs 11%; *P*=0.009), proinsulin peptides: C13-32, (55 vs 11%; *P*=0.0004), C19-A3 (42 vs 14%; *P*=0.02) and C22-A5 (27 vs 4%; *P*=0.0001) and GAD 555-67 (30 vs 4%; *P*=0.007) were all significantly higher in children (Fig. 1b). By contrast, responses to individual peptides were detected in 2–8% of the younger siblings and 2–11% of the adult autoantibody-negative siblings without Type 1 diabetes.

The breadth of the IFN-γ response, as measured by the number of peptides an individual responded positively against, differed significantly between children and adults with Type 1 diabetes: only 4/28 (14%) adults showed a response to three or more islet peptides, whereas this was observed in 23/33 (70%) children (*P*<0.0001). The median number of peptides eliciting an IFN-γ response in children was higher: 4 vs 1 in children vs adults (*P*<0.0001) (Fig. 1c); thus, at diagnosis of Type 1 diabetes, children have IFN-γ responses to multiple islet peptides more frequently than adults.

The magnitude of the autoreactive response was assessed in subjects who had positive peptide-specific IFN-γ responses. The magnitude of the response to each peptide was similar in children and adults; however, differences were observed for insulin peptide B1-20 and IA-2 752-775 (mean SI higher in children; *P*=0.0009 and *P*=0.01, respectively; Fig. 1d). The magnitude of the response to pentavalent vaccine was not significantly different across the study groups (Table 1).

Interleukin-10 responses in children and adults with newly diagnosed Type 1 diabetes

In contrast to IFN-γ responses, the frequency of people having an IL-10 response to islet-autoantigen peptides was similar in children (19/33, 58%) and adults (13/28, 46%; *P*=0.4) with Type 1 diabetes (Fig. 1a) and unaffected siblings [31/48 children (65%); 30/43 adults

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(70%)] without Type 1 diabetes. Again, there was no gender bias, and the prevalence of IL-10 responses to pentavalent vaccine was similar in children and adults (96 and 97%, respectively).

The specificity of the IL-10 response to individual peptides was varied and showed no distinct pattern (Fig. 1e). The breadth of the IL-10 response was not significantly different between children and adults with Type 1 diabetes; 5/28 (18%) adults showed a response to three or more islet peptides compared with 11/33 (33%) children (P=0.2). The median number of peptides eliciting an IL-10 response was 1 in children and 0 in adults (Fig. 1c). The magnitude (SI value) was not significantly different between children and adults (Fig. 1f) and pentavalent vaccine responses were similar across groups. The IL-10 response to pentavalent vaccine was similar in all groups (Table 1).

Overall, the responses in children with Type 1 diabetes show an IFN-γ predominance (Fig. 2a): 13/33 children (39%) showed an exclusive IFN-γ response compared with 4/33 (12%) with IL-10 response only (P=0.02). Such skewing was not observed in adults [9/28 (32%) for IFN-γ and 6/28 (21%) for IL-10; P=0.5]. When examining only insulin- and proinsulin-specific responses, which were far more prevalent in children, this pro-inflammatory polarization was much more apparent: 81% of children (27/33) had an IFN-γ response compared with 39% of adults (11/28; P=0.0012). IL-10 responses to proinsulin and insulin peptides were similar in children (55%,18/33) and adults (46%,13/28; P=0.6)

None of the autoimmune phenotypes were influenced by the presence of HLA-*DRB1*0301* and **0401* genotypes in either children or adults with Type 1 diabetes (data not shown).

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Autoimmune phenotypes in children and adults with Type 1 diabetes

We agglomerated peptide-specific responses into their parent antigens, and analysed these alongside autoantibodies (Fig. 2b). The prevalence of autoantibodies in children and adults was similar; however, IFN-γ responses against proinsulin and insulin were significantly more frequent in children (70 and 63%, respectively) compared with adults (18 and 36%, respectively; $P<0.0001$ and $P=0.04$). Also notable was a significantly lower GAD65-specific IL-10 response in adults (12.5%) compared with children (36%; $P=0.03$).

Autoantibody and T-lymphocyte responses

Of the people positive for GAD autoantibodies, a corresponding T-lymphocyte inflammatory (IFN-γ) response was seen in more children than in adults [17/20 (85%) vs 11/21 (52%); $P=0.04$]. For IA-2 autoantibody positivity, a corresponding T-lymphocyte inflammatory response was seen in more children than in adults [23/25 (92%) vs 8/16 (50%); $P=0.007$]. For ZnT8 autoantibody positivity, there was a trend for corresponding T-lymphocyte inflammatory response to be greater children than in adults but this was not statistically significant [19/22 (86%) vs 9/16 (56%); P = non-significant]. By contrast, there was no relationship between any autoantibody positivity and IL-10 responses. Overall, the stronger relationship between autoantibodies and inflammatory T-lymphocyte responses in children emphasizes the stronger pro-inflammatory bias in the young.

Discussion

The present study compares islet antigen-specific cellular immune responses in recent-onset Type 1 diabetes arising in childhood and adult life; and has led to a novel observation: near to diagnosis of Type 1 diabetes, pro-inflammatory autoreactivity is significantly more prevalent,

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and targets a wider range of islet peptides in children than adults, which is consistent with a broader and thus more aggressive autoimmune response in the younger age group. This finding is consistent with the proposition that islet autoreactivity is broader and more aggressive (or less well regulated) in the younger age group. In younger subjects, epitopes of proinsulin and insulin were also preferentially targeted, suggesting that the antigenic driver(s) of disease also differ with age. We speculate that these findings are linked to, and provide a mechanistic explanation for the known tendency of C-peptide reserve to decline at a faster rate in younger people after Type 1 diabetes diagnosis [10].

The increased frequency of IFN-γ responses in children could be attributable to several different influences, which will need to be examined in future studies; control of autoreactivity by naturally arising CD4+CD25^{hi}FoxP3+CD127^{lo} regulatory T cells (nTregs) could differ between adults and children; indeed lower numbers of T regs have been reported in children with Type 1 diabetes [11,12]. Furthermore, it has been shown previously that there is a correlation between increasing age and frequency of nTregs in Type 1 diabetes [13] and it is conceivable that, as a consequence, adaptive immune regulation is stronger in adulthood, leading to a more limited autoreactive T cell response. A further possibility is that the lower observed autoreactivity in adults reflects a relatively low genetic load of Type 1 diabetes predisposing genotypes [14], some of which are likely to influence disease susceptibility via effects on immune regulation of effector pathways. In a recent study of people aged ≥17 years at diagnosis, the slower progression toward autoimmune insulin deficiency was ascribed to a lower Type 1 diabetes-predisposing genetic load [14]. Also the same authors noted that non-HLA genes conferring susceptibility were associated with a lower age of diagnosis [15].

Interestingly, although the frequency and breadth of the pro-inflammatory autoimmune response was greater in children in the present study, the magnitude of the response as measured by SI was generally similar in children and adults. The SI acts as a surrogate for the

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number of responder cells, suggesting that what marks out children developing Type 1 diabetes is a polyspecific response that targets more autoantigens and/or more epitopes, in keeping with the notion that determinant spreading is a key immunological driver [16].

We further explored this response to proinsulin and insulin peptides and demonstrated significantly higher IFN-γ responses in children compared with adults; IL-10 responses did not differ between the two groups. This is an interesting finding and suggests a polarization, with a pro-inflammatory response against peptides of proinsulin and insulin specifically in children, which has major implications for choice of immunotherapy in this population.

Responses characterized by release of the natural immune suppressive cytokine IL-10 did not differ between children and adults, apart from with respect to GAD65, which was significantly less frequent in adults. It is tempting to speculate that this relatively poor GAD65-specific immune regulation is related to the greater propensity for Type 1 diabetes in adults to focus on GAD65 as a major autoantigen for autoantibody responses [17].

There are some caveats and limitations to the present study. For example, future studies in cohorts followed longitudinally will be needed to address whether age-related differences in T-cell responses are persistent and have the same behaviour for other autoantigens, and whether they are influenced by high-risk HLA alleles of *DQA1* and *DQB1* genes and the extent to which they relate directly to rate of disease progression and loss of C-peptide. Larger numbers of subjects could also explore how responses to other autoantigens differ, especially in those people with Type 1 diabetes and an additional autoimmune disease. Although not significant, we observed a trend for vaccine-specific IL-10 and IFN-γ responses to be lower in adults. We speculate that this reflects the distance in years that adults are from exposure to these recall antigens in vaccines or wild-type infections, compared with the children who

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would have been actively immunized more recently, and this should be explored in future studies.

It would also be of interest to see whether these distinct phenotypes were present during preclinical disease, as such studies have not been conducted.

The present study provides evidence to substantiate the hypothesis that the autoimmune response in children developing Type 1 diabetes is more pro-inflammatory and less regulated than in adults, further highlighting recent reports of heterogeneity in disease pathogenesis [7]. Viewed from the perspective of designing intervention trials and selecting therapeutic agents, our findings suggest that these may require greater attention to age/inflammatory set-point than has been the case hitherto.

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Competing interests

None declared.

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FIGURE 1 Frequency and magnitude of interferon (IFN)-γ and interleukin (IL)-10 responses in Type 1 diabetes according to age at disease diagnosis. (a) The frequency of IFN-γ and IL-10 responses in children (open bars) and adults (shaded bars) with Type 1 diabetes. Bars

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represent means of percent responders to any peptide (*P=0.04). (b) Stacked bars showing the prevalence of IFN-γ responses to each islet autoantigenic peptide in children (open bars) and adults (shaded) with Type 1 diabetes. The frequency of responses to each peptide was compared using Fishers exact test and P values of <0.05* and P<0.0005** are shown. (c) Scatter plot represent the number of peptides eliciting IFN-γ and IL-10 responses in children (open squares) and adults (black circles) with Type 1 diabetes; the median response is represented by the black horizontal line (***P<0.001). (d) The mean stimulation index (SI) values for each peptide response in children (open squares) and adults (black circles) with Type 1 diabetes for IFN-γ responses. The frequency of responses to each peptide has been compared by an unpaired *t*-test and P values of <0.05* and P<0.005**** are shown. (e) Stacked bars showing the prevalence of IL-10 responses to each islet autoantigenic peptide in children (open bars) and adults (shaded) with Type 1 diabetes. (f) The mean SI value for each peptide response in children (open squares) and adults (black circles) with Type 1 diabetes for IL-10 responses. GAD, glutamic acid decarboxylase antibodies; IA-2, insulinoma-associated antigen 2.

FIGURE 2 The autoimmune response is skewed towards a pro-inflammatory phenotype in children and peptides of proinsulin and insulin are preferentially targeted by this pro-inflammatory immune response. (a) Autoreactive T-cell responses to β-cell peptides in children (open red triangles) and adults (open blue circles). Positive peptide responses [stimulation index (SI) >3 for interferon (IFN)-γ and/or interleukin (IL)-10] have been plotted for each cytokine; the numbers in each quadrant represent number of positive responses. (b) CD4 T-cell responses to islet target peptides agglomerated according to parent antigen. Graph shows frequency of response to islet autoantigens in children (*x*-axis) and adult (*y*-axis). Red circles denote IFN-γ responses, blue circles IL-10 responses, and green circles autoantibody responses. Filled circles indicate a statistically significant difference (P< 0.05*) in the

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frequency of responses between the two groups. Grey lines are 95% CIs. Ins, insulin; PI, proinsulin. GAD, glutamic acid decarboxylase antibodies; IA-2, insulinoma-associated antigen 2.

Table 1 Demographic data on people with Type 1 diabetes and unaffected siblings

	Adults ***n* = 28**	**Unaffected siblings: adults** ***n* = 43**	**Children** ***n* = 33**	**Unaffected siblings: children** ***n* = 48**
Median (range) duration of Type 1 diabetes, weeks	8 (4–12)	N/A	7 (4–12)	N/A
Median (range) age, years	30 (17–42)	22 (17–38)	12 (8–16)	13 (6–16)
Males, %	71	47	55	38
Frequency of HLA genes (%)				
*DRB1*0301*	9/28 (32)	13/43 (30)	9/33 (27)	17/48 (35)
*DRB1*0401*	19/28 (68)	30/43 (70)	24/33 (73)	31/48 (65)
Mean ± SEM HbA_{1c}				N/A
mmol/mol	65.6 ± 5.0	N/A	67.35 ± 7.6	
%	8.2 ± 2.6		8.3 ± 2.8	
Autoantibodies (%)				
GAD antibodies	21/28 (75)	N/A	20/33 (60)	N/A
IA-2 antibodies	16/28 (57)	N/A	25/33 (76)	N/A

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ZnT8 antibodies	16/28 (57)	N/A	22/33 (67)	N/A
Recall responses to pentavalent vaccine, %				
Prevalence of IFN-γ responses	94	100	97	96
Prevalence of IL-10 responses	97	100	97	98

GAD, glutamic acid decarboxylase antibodies; IA-2, insulinoma-associated antigen 2; IFN, interferon; IL, interleukin; N/A, not applicable; ZnT8, zinc transporter 8.

Figure 1

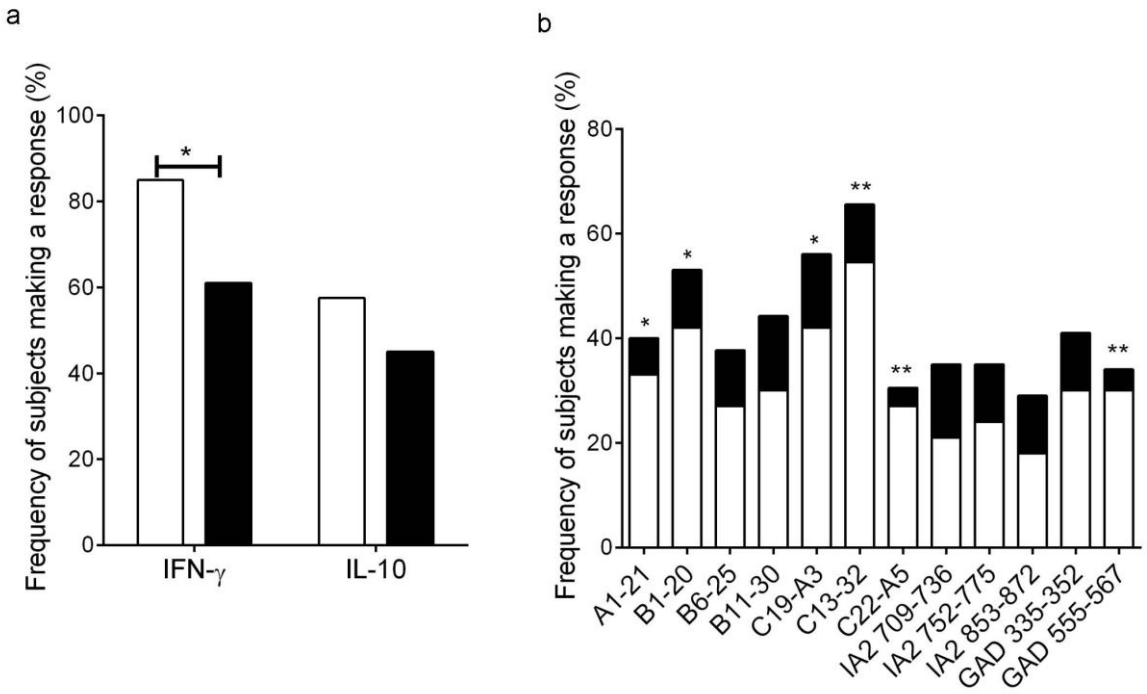

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Figure 1

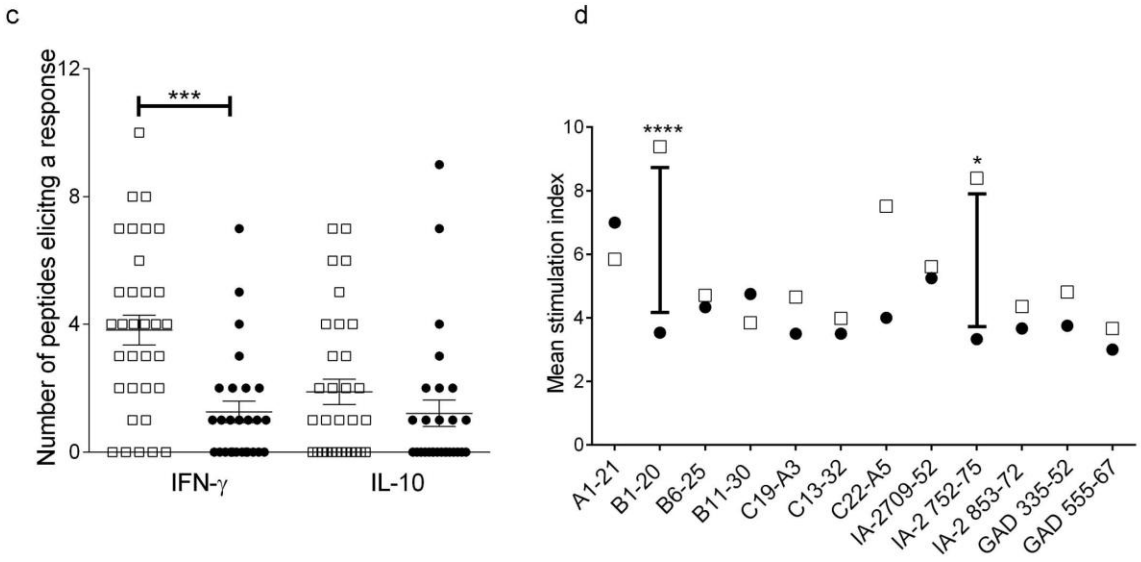

Figure 1

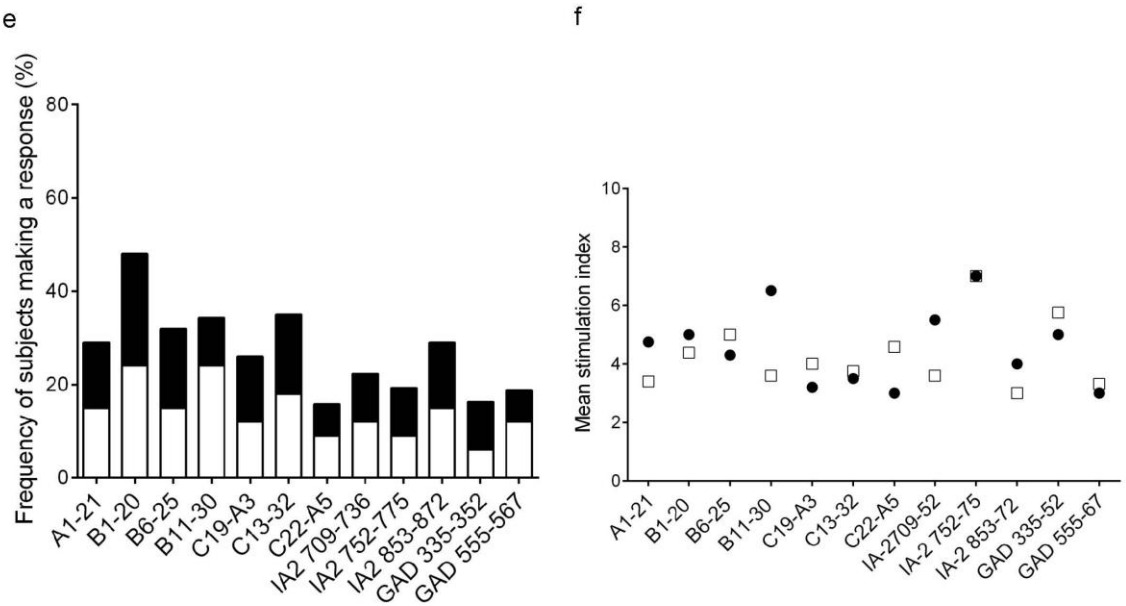

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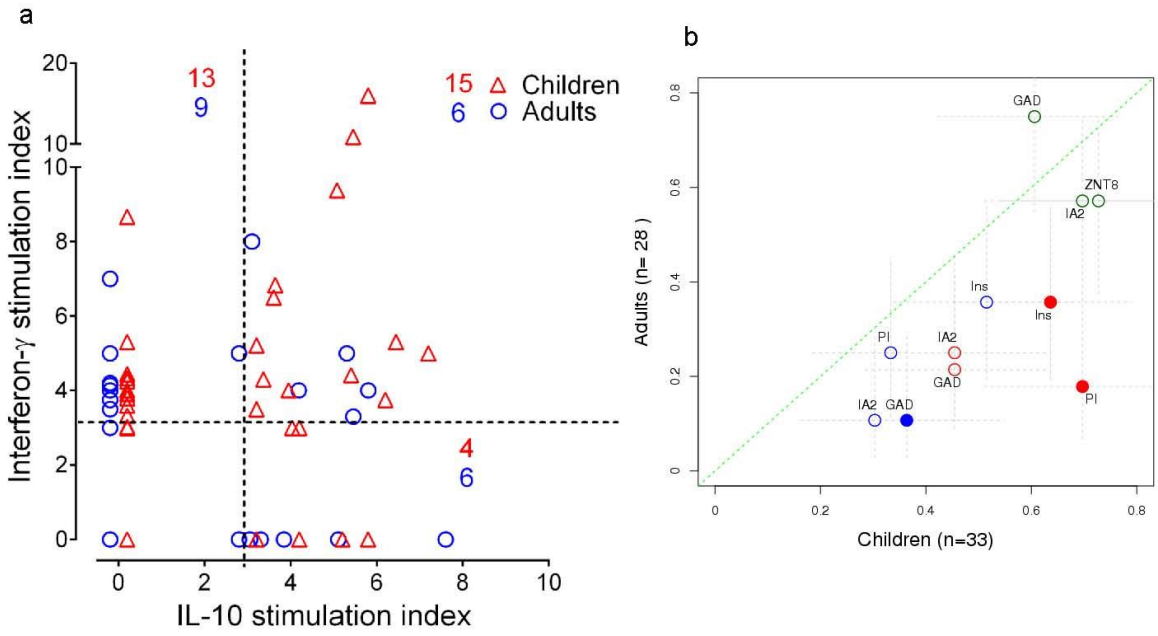
Figure 2



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